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Abstract

In the context of a study on the occurrence of *Listeria* spp. in farm animal environment in the region of Valencia, Spain, six *Listeria*-like isolates were isolated and could not be assigned to any known species. Phylogenetic analysis based on the 16S rRNA gene and on 231 *Listeria* core genes grouped these isolates in a monophyletic clade within the *Listeria* genus*,* with highest similarity to *Listeria thailandensis*. Whole-genome sequence analyses based on *in silico* DNA-DNA hybridization, the average nucleotide BLAST and the pairwise amino acid identities against all currently known *Listeria* species confirmed that these isolates constituted a new taxon within *Listeria* genus. Phenotypically, these isolates differed from other *Listeria* species mainly by the production of acid from inositol, the absence of acidification in presence of methyl α-D-glucoside, and the absence of α-mannosidase and nitrate reductase activities. The name *Listeria valentina* sp. nov. is proposed for this novel species, and the type strain is CLIP 2019/00642^T (=CIP 111799^T=DSM 110544^T).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and draft genome of strain CLIP 2019/00642^T are MN686588 and JAATJW000000000, respectively.

Main text

The screening of environmental *Listeria* isolates and the use of whole-genome based identification methods contribute to obtain a better picture of the diversity and world distribution of the *Listeria* genus. Twenty species have been described so far, which can be subdivided in two major groups [1-3]: (i) *Listeria sensu stricto*, constituted by *Listeria monocytogenes* [4], *L. innocua* [5], *L. welshimeri* [6], *L. seeligeri* [6], *L. ivanovii* [7] and *L. marthii* [8], and (ii) *Listeria sensu lato*, constituted by *L. grayi* [9, 10], *L. rocourtiae* [11], *L. fleischmannii* [12, 13], *L. weihenstephanensis* [14], *L. floridensis* [15], *L. aquatica* [15], *L. cornellensis* [15], *L. riparia* [15], *L. grandensis* [15], *L. booriae* [16], *L. newyorkensis* [16], *L. costaricensis* [17], *L. goaensis* [18] and *L. thailandensis* [19]. Only two species, *L. monocytogenes* and *L. ivanovii* subsp. *ivanovii,* are pathogenic for human and ruminants [20].

During a study on the occurrence of *Listeria* species in farming environment in South-East Spain (Valencia) in 2018 and 2019, six isolates of *Listeria*-like bacterium were isolated from the faeces of healthy sheep (*Ovis aries* breed Lacaune) and surrounding water trough (Table 1). Rectal faecal samples were obtained by veterinarians during usual handling of the animals, in accordance with the guidelines of European Union Directive 2010/63/EU for the protection of animals used for scientific purposes [21]. This routine veterinary practice does not require the approval of the Animal Ethics and Experimentation Committee. Faecal samples (8 g) were diluted 1/10 in Half-Fraser broth (Scharlab, Barcelona, Spain) and incubated at 30°C for 24 h for enrichment. Swab samples were taken from environmental surfaces (water trough), placed in 10 ml Half Fraser broth, vortexed for 2 min and incubated at 30°C for 24 h for enrichment. One hundred microliters of the incubated suspension were transferred to 10 ml of Fraser broth (Scharlab) and incubated at 37 °C for 24 h. After the second incubation, 100 µl of enriched culture and two ten-fold dilutions were transferred to RAPID'*L.mono* plates (BioRad, Marne-la-Coquette, France) and incubated at 37°C for 24 h. Typical *Listeria* sp. colonies (i.e. white round colonies of 1 to 2 mm diameter, with or without a pale yellow halo) were confirmed in selective Oxford agar plates for *Listeria* (Scharlab). On Oxford agar, colonies were approximately 2 mm in diameter, grey-green in colour with a black sunken centre and a black halo. The six presumptive isolates of *Listeria* sp. (Table 1) were sent to the World Health Organization Collaborating Centre (WHOCC) for *Listeria*, Institut Pasteur, Paris, France, for species identification and characterization.

Isolates were grown on Brain Heart Infusion agar plates (BHI, Becton Dickinson), and on two chromogenic media: i) the Rapid'*L.mono* agar (BioRad), which detects phosphatidylinositol-specific phospholipase C (PI-PLC) through the hydrolysis of X-inositol phosphate and the

fermentation of D-xylose, and ii) the Agar *Listeria* according to Ottaviani and Agosti (ALOA; bioMérieux, Craponne, France), which simultaneous detects PI-PLC and β-glucosidase activities. On BHI agar, after 24 h and 48 h at 30 and 37 °C, colonies were opaque, flat and with a diameter of 0.5 to 1.0 mm. On Rapid'*L.mono*, after 24h at 37 °C, colonies were round, convex, 1 to 2 mm, white and surrounded by a pale yellow halo due to fermentation of D-xylose [22]. On ALOA agar, after 24 h at 37 °C, colonies were round, smooth-edged and blue, due to β-glucosidase activity, and were not surrounded by a white halo, indicating the absence of PI-PLC activity (Table 2) [23].

Species identification using MALDI-TOF mass spectrometry using the MicroFlex LT system with the MBT library DB-7854 (Bruker Daltonics, Bremen, Germany), which comprises all species of *Listeria,* except *L. marthii, L. costaricensis, L. goaensis, L. rocourtiae and L. thailandensis* [24], was inconclusive, but assigned these isolates as members of the genus *Listeria* (scores between 1.73 and 1.99). The API *Listeria* (bioMérieux API web database, version 2.0) biochemical systems also failed to provide any reliable identification (API numerical profile 2731, as the recently described *L. thailandensis* [19]).

DNA sequences were obtained from Illumina DNA sequences were obtained using Illumina NextSeq 500 and 2 x 150 bp paired-end reads. Raw reads were trimmed with fqCleaner v.3.0 (Alexis Criscuolo, Institut Pasteur) as previously described [25]. Trimmed reads (final coverage >70X; Table 1) were assembled and polished with the Unicycler v0.4.4 pipeline [26], using SPAdes v.3.7.0 [27], Bowtie2 v2.2.9 [28] and Pilon v.1.22 [29]. Contigs smaller than 500 bp were discarded from the final assemblies. Data obtained followed the quality standards for its use for taxonomic purposes [30]. Draft assemblies were annotated with Prokka v.1.12 [31] and screened for the presence of plasmids using MOB-suite v.2.0.1 [32]. The core genome alignment of the six isolates was built using Parsnp, implemented in Harvest v.1.1.2 suite [33], using CLIP 2019/00642^T genome as reference and enabling the filtering of recombination regions. Draft assemblies had a total length ranging from 2.68 to 2.75 Mb (Table 1) and an average G+C content of 40.2 mol% similar to other members of the genus *Listeria* (36.35%–43.71 mol%). No plasmids were detected. Isolates differed from CLIP 2019/00642^T by 128 to 245 nucleotides out of 2,536,866 core genome alignment positions (Supplementary Fig. S1A). The six isolates comprised a total of 2,801 predicted gene families, 2,498 of which common to all isolates (Supplementary Fig. S1B), as defined using Roary v.3.12 [34] and a BLASTP identity cut-off of 95%.

Phylogenetic analyses at the genus level were performed based on 16S rRNA gene sequences and on the concatenated deduced amino acid sequences of 231 core genes present in all *Listeria* species [19], defined using Roary v.3.12 [34] and a BLASTP identity cut-off of 80%. Sequences were aligned using MUSCLE v.3.8 [35]. Maximum likelihood phylogenetic trees were inferred by using IQ-Tree v.1.5 [36], using the Kimura 2-parameter [37] and the Whelan and Goldman (WAG) [38] substitution models to compute nucleotide and amino acid distances, respectively, and visualized in MEGA v.7.0 [39]. Both 16S rDNA sequence (Fig. 1) and core genome (Fig. 2) analyses showed highest sequence similarity to *L. thailandensis*. Interestingly, 16S sequence similarity with *L. thailandensis* was of 99.4%, above the proposed species cut-off based on 16S sequence similarity (98.7-99.0%; [40, 41]).

Average nucleotide identities (ANI) against *Listeria* species were determined using the enveomics package [42], using the BLASTN settings defined as in JSpecies v1.2.1 and Goris *et al.* 2007 [43] (Blastall v. 2.2.26, dropoff value for gapped alignment X=150 bits, penalty for nucleotide mismatch q=−1, e-value ≥ 1e-15, identity ≥ 70%, alignment length ≥ 70%). Amino acid identities (AAI) were determined using the enveomics package [42] using default parameters. Dendrograms based on the unweighted pair group method with arithmetic mean (UPGMA) method were obtained from the ANI and AAI distance matrixes using Bionumerics v.7.6 (Applied Maths, Sint-Martens-Latem, Belgium). *In silico* DNA–DNA hybridization similarities (isDDH), which closely reflect experimentally derived DDH values [44], were calculated using the GGDC 2.1 web server and formula 2, which takes into account the sum of all identities found in high-scoring segment pairs (HSP) divided by overall HSP length [45, 46], The pairwise percentage of conserved proteins (POCP) was determined as described [47]. All six isolates, including the type strain CLIP 2019/00642^{T} (originally designated as LISVAL109) shared less than 95% genome sequence identity with its closest relative *L. thailandensis* (two-way ANI of 93.61%±3.37%, based on 9,805 genome fragments) (Fig. 3, Supplementary Table 1), lower than the proposed genomic cut-off for identification of bacterial species [43]. The average isDDH was of 54.8% [51.9-57.4%] between *L. valentina* isolates and *L. thailandensis*, well below the 70% cut-off recommended for the delineation of species [46]. Analyses based on the deduced proteomes also showed highest identities with *L. thailandensis* (two-way AAI of 95.47±9.32%, based on 2241 orthologous proteins; POCP of 84.1%). Both AAI and POCP pairwise analyses were higher than proposed cut-offs for genus delineation of 60% and 50%, respectively [42, 43]. Taken together, these results confirm that these six isolates belong to a novel taxon within *Listeria* genus. The name *Listeria valentina* is proposed for this novel species.

Isolates were then characterized at the phenotypic level (Table 2 and Supplementary Fig. 2) as described below. Unless specified otherwise, all inocula were grown on BHI agar at 30 °C for 24 h.

Gram staining was performed with the Color Gram 2 kit (bioMérieux), according to the manufacturer's instructions. Catalase and oxidase activities were determined using the API ID Color catalase kit (bioMérieux) and the Bactident oxidase test strips (Merck Millipore, Molsheim, France), respectively, according to the manufacturer's instructions. Respiratory characteristics were determined by growth on BHI agar plates at 30°C after 48 h in aerobic incubator and in anaerobic jars containing an anaerobic catalyst according the manufacturer's instructions (Anaerocult A; Merck Millipore), as described by Lang Halter *et al.* [48]. Endospore staining was performed using the Schaeffer-Fulton's method, as described by Hussey and Zayaitz [49, 50]. The presence of capsule was determined using the Duguid wet-Mount method with nigrosin (Sigma-Aldrich, Saint-Louis, USA), as previously described [51]. All *L. valentina* bacilli were Gram-stain-positive facultative anaerobic rods, catalase positive, oxidase negative and produced no capsule or endospores, in agreement with the main characteristics of the genus *Listeria* [2].

Motility was tested by stab-inoculation in mannitol-mobility semi-solid agar (Bio-Rad, Marnes-La-Coquette, France), followed by incubation at different growth temperatures (4°C, 22°C and 37°C) for 10 days in aerobic conditions. *L. monocytogenes* ATCC 35152^T and *L. booriae* CIP 111022^T were used as positive and negative controls, respectively. None of *L. valentina* isolates were motile at any tested temperature.

Classical serotyping was performed against somatic (O; I to XV) and flagellar (H; A to E) antigens as described by Seeliger and Höhne [52], using with commercial factor sera (I to IX and A to D antigens; Denka Seiken, Tokyo, Japan) and reference factor sera from the WHOCC *Listeria* (X to XV and E antigens; Institut Pasteur, Paris) [52]. *L. valentina* isolates were agglutinated by sera V and XV targeting known somatic antigens, and did not react with sera directed against flagellar antigens, confirming the absence of motility of these isolates. PCR-serogrouping determined by multiplex [53] and *in silico* from draft assembled genomes as described previously [53-55] was positive for the *prs* gene, corresponding to genoserogroup L (typical of *Listeria* species with the exception for *L. monocytogenes* [53]).

Haemolysis was determined by stabbing isolates into Columbia agar plates (bioMérieux) containing 5% defibrinated blood of either sheep or horse, as described in the *Bacteriological Analytical Manual* (BAM) [49], following by incubation for 24h at 37°C. The Christie, Atkins,

Munch-Petersen (CAMP) test was performed as previously described [49, 56]. Briefly, isolates and controls were streaked horizontally on Columbia agar containing 5% defibrinated sheep blood (bioMérieux), together with *Staphylococcus aureus* CIP 5710 and *Rhodococcus equi* NCTC 1621, which were streaked vertically. Plates were incubated at 37 °C for 24-48 h and examined for haemolysis in the zone of influence of the vertical streaks. *L. monocytogenes* ATCC 19115, *L. ivanovii* ATCC 19119, *L. seeligeri* ATCC 35967 and *L. innocua* ATCC 33090 were used as controls. Haemolysis and CAMP tests were negative for all *L. valentina* isolates. The lack of haemolysis was consistent with the absence of *Listeria* pathogenicity islands (LIPI) [57, 58] within *L. valentina* draft genomes, suggesting that this novel taxon is likely not pathogenic for humans and animals.

Growth characteristics were determined on BHI agar and broth at 22, 30, 37 and 42 °C for 7 days and at 4 °C for 10 days. Colonies on BHI agar were difficult to homogenise in BHI broth due to the formation of culture flakes. Growth was considered positive if there was an increase in cell number of at least 1.0 log ($cfu.ml^{-1}$). All isolates showed growth between 22 and 42 °C but not at 4 °C.

Nitrate reduction was determined as described in the BAM [49], by inoculation in nitrate broth (bioMérieux) and incubation at 37°C for 5 days. In the absence of a red-violet colour development after addition of Nitrate 1 and Nitrate 2 reagents (bioMérieux), which indicated that nitrate had not been reduced to nitrite, powdered zinc (bioMérieux) was added to the solution and incubated at room temperature for 1 hour. The developing red-violet colour indicated that nitrate was still present and has not been reduced. *L. fleischmannii* CIP 110547^T and *L. monocytogenes* ATCC 19115 were used as a positive and negative controls, respectively. The incapacity to reduce nitrate and nitrite were observed for all six isolates (Table 2).

The Voges-Proskauer test, which detects the presence of acetoin, a precursor of 2,3 butanediol, was performed by inoculation on Voges-Proskauer medium (Bio-Rad), and incubation at 37°C. After 48 h, α-naphthol VP1 and KOH VP2 solutions (bioMérieux) were added, as described in the BAM [59] and incubated at room temperature for 10 minutes. *L. fleischmannii* CIP 110547^T and *L. monocytogenes* ATCC 19115 were used as a positive and negative controls, respectively. The absence of a pink-to-ruby red colour observed for all six isolates indicated a negative Voges-Proskauer test (Table 2).

Biochemical tests were performed in triplicates with API *Listeria* strips (bioMérieux) and the API50CH system (bioMérieux) as recommended by the manufacturer [60]. API *Listeria* tests

were recorded after incubation at 37 °C for 24h. API50CH tests were recorded after 2, 5, 10 and 15 days of incubation at 37 °C. Results are shown in Table 2 and Supplementary Fig. S2. Comparing to other *Listeria* species, all *L. valentina* isolates could be readily distinguished by the production of acid from inositol, by the absence of production of acid from methyl α-D-glucoside and by the absence of α-mannosidase and nitrate reductase activities. Differences among the six isolates were only observed for the utilization of D-tagatose. Whereas isolate CLIP 2019/00570 was D-tagatose negative in API50CH but positive in API *Listeria* (API numerical profile 2731), all other five isolates (including the type strain CLIP 2019/00642^T) were D-tagatose positive in both systems. All isolates carried 3 genes encoding tagatose 1,6-diphosphate aldolases, with the exception of CLIP 2019/00570, which carried only 2 of these genes. The absence of a third tagatose 1,6-diphosphate aldolase gene and the different composition of reconstituted media in identification strip systems could account for these results.

Finally, susceptibility to antimicrobials was determined with the disk diffusion method on Mueller–Hinton agar plates (Bio-Rad), using the interpretative criteria and recommendations from the CA-SFM (freely available at https://www.sfm-microbiologie.org/wp-content/uploads/2019/02/CASFM2019_V1.0.pdf) and the European Committee on Antibiotic Susceptibility Testing (EUCAST, freely available at https://www.eucast.org/). The following antibiotic discs (Bio-Rad) were used: amoxicillin (25 µg), ampicillin (10 µg), cefotaxime (30 µg), sulphonamides (200 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (15 µg), fosfomycin (50 µg), fusidic acid (10 µg), gentamicin (15 µg), imipenem (10 µg), kanamycin (30 µg) moxifloxacin (5 µg), nalidixic acid (30 µg), penicillin G (6 µg), rifampicin (30 µg), streptomycin (10 µg), tetracycline (30 µg), trimethoprim (5 µg), and vancomycin (30 µg). The diameters of inhibition zones were measured with Scan 4000 (Interscience, Saint-Nom-la-Bretèche, France). *L. valentina* isolates were sensitive to penicillin G, ampicillin, amoxicillin, imipenem, kanamycin, streptomycin, gentamicin, rifampicin, erythromycin, levofloxacin, moxifloxacin, tetracycline, chloramphenicol, fusidic acid, ciprofloxacin, fosfomycin, trimethoprim and vancomycin, and resistant to nalidixic acid and clindamycin, as other *Listeria* species [61]. Of note, all isolates were borderline resistant to cefotaxime (equal to 25 mm diameter cutoff value), in contrast to other *Listeria* species which are unambiguously resistant. With the exception for CLIP 2019/00569 and CLIP 2019/00570, all other *L. valentina* isolates were resistant to sulphonamides. At the genomic level, only genes conferring resistance nalidixic acid (*norB*) were detected in *L. valentina* draft assemblies. Known genes conferring tolerance towards antiseptics or detergents were absent [55].

On the basis of the phenotypic distinctiveness and the molecular findings described above, we propose that these isolates are classified as a novel species of the genus *Listeria* for which we propose the name *Listeria valentina* sp. nov.

DESCRIPTION OF *LISTERIA VALENTINA* SP. NOV.

Listeria valentina (va.len.ti.na N.L. fem. adj. *valentina*, "from Valencia", the region where the type strain was isolated).

Cells are straight, Gram-stain-positive, non-motile and non-spore-forming rods. Facultative anaerobic, catalase-positive and oxidase-negative. Capsule is not formed. Colonies are opaque, not pigmented, with a flat shape and entire margin on BHI. On ALOA, colonies are blue centred without white halo, typical of *Listeria* species. Growth occurs at 22-42 °C, with optimal growth at 30-37 °C. Negative for haemolysis, nitrate and nitrite reduction and the Voges-Proskauer test. Absence of the enzymes D-arylamidase, α-D-mannosidase and phosphatidylinositol-specific phospholipase C. Acid is produced from aesculin, N-acetylglucosamine, amygdalin, arbutin, D-cellobiose, D-fructose, gentiobiose, D-glucose, glycerol, D-mannose, L-rhamnose, D-ribose, salicin, D-trehalose, D-tagatose, D-lyxose, L-arabinose, D-arabitol, inositol and D-xylose. Acid is not produced from D-adonitol, D-arabinose, L-arabitol, L-fucose, D-lactose, D-maltose, dulcitol, D-saccharose, D-galactose, methyl α-D-glucoside, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, methyl β-D-xylopyranoside, potassium gluconate, potassium 5-ketogluconate, glucose 1-phosphate, xylitol, starch, erythritol, D-fucose, glycogen, inulin, D-mannitol, D-melezitose, D-melibiose, potassium 2-ketogluconate, D-raffinose, D-sorbitol, L-sorbose, D-turanose, L-xylose. It can be differentiated from other *Listeria* species by the production of acid from inositol, the absence of production of acid for methyl α-D-glucoside, the absence of the enzyme α-mannosidase and nitrate reductase.

The type strain CLIP 2019/00642^T was isolated in February 2019 from a healthy sheep faeces sample in the region of Valencia, Spain. The genomic DNA G+C content of the type strain is 40.2 mol%. The type strain is deposited at the Leibniz Institute DSMZ – German Collection of Microorganisms (DSM 110544^T) and at Collection of Institut Pasteur (CIP 111799^T). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and draft genome of strain CLIP 2019/00642^T are MN686588 and JAATJW000000000, respectively. All sequence data is available under BioProject PRJEB36008.

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Conflicts of interest

The authors declare no conflict of interests.

Abbreviations:

AAI	Average amino acid identity
ANI	Average nucleotide identity
BAM	Bacteriological Analytical Manual
BHI	Brain hearth infusion
BLAST	Basic local alignment search tool
CAMP	Christie, Atkins, Munch-Petersen test
isDDH	*In silico* DNA–DNA hybridization
N50	Minimum contig length covering 50% of the genome
POCP	Pairwise percentage of conserved proteins
UPGMA	Unweighted pair group method with arithmetic mean

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Table 1. Characteristics and genome metrics of the *L. valentina* strains reported in this study.

isolate	original name	isolation year	source	serogroup	remarks	no. paired reads[a]	average coverage	no. contigs	N50 contig no.	N50 contig length (Kb)	total length (Mb)	%GC	NCBI-SRA accession no.	#BIGSdb[b]
CLIP 2019/00642[c]	LISVAL109	2019	Healthy sheep faeces	L	species type strain	8.02E+05	95	122	19	43.2	2.68	40.2	ERR3865670	48019
CLIP 2019/00569	LISVAL106	2019	Healthy sheep faeces	L		1.06E+06	119	136	17	45.2	2.73	40.1	ERR3865665	48020
CLIP 2019/00570[d]	LISVAL107	2019	Healthy sheep faeces	L	tagatose negative	8.15E+05	92	128	15	52.8	2.68	40.2	ERR3865666	48021
CLIP 2019/00571	LISVAL108	2019	Healthy sheep faeces	L		9.15E+05	105	126	18	45.2	2.75	40.1	ERR3865667	48022
CLIP 2019/00572	LISVAL110	2019	Healthy sheep faeces	L		1.13E+06	128	141	17	43.2	2.73	40.1	ERR3865668	48023
CLIP 2019/00574[e]	LISVAL112	2019	Water trough	L		6.31E+05	71	151	24	30.6	2.69	40.3	ERR3865669	48024

[a] after trimming for low quality reads

[b] https://bigsdb.pasteur.fr/listeria/ [55]

[c] Strain number CIP 111799 and DSMZ 110544

[d] Strain number CIP 111800 and DSMZ 110545

[e] Strain number CIP 111801 and DSMZ 110546

Table 2. Biochemical characteristics of species of the genus *Listeria* based on observations made in this study and on previously published studies [15, 17-19].

Characteristics	*Lmo*	*Lin*	*Lse*	*Liv*	*Lws*	*Lma*	*Lgy*	*Lro*	*Lwp*	*Lcn*	*Lri*	*Lgd*	*Lfc*	*Laq*	*Lfo*	*Lny*	*Lbo*	*Lco*	*Lgo*	*Lth*	*Lva**
Motility	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Nitrate reduction	−	−	−	−	−	−	v	+	+	+	+	+	+	+	−	+	+	+	−	+	−
Voges-Proskauer	+	+	+	+	+	+	+	−	−	−	−	−	−	v	−	−	−	+	−	+	−
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
Haemolysis	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+ (α)	−	−
D-Arylamidase	−	+	+	v	v	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
α-Mannosidase	+	+	−	−	+	+	v	+	−	−	+	−	−	+	−	−	+	−	−	−	−
Phosphatidylinositol-specific phospholipase C (PI-PLC)	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Acidification of:																					
D-Arabitol	+	+	+	+	+	+	+	−	+	−	−	v	+	−	−	−	+	+	+	+**	+**
D-Galactose	v	−	−	v	−	−	+	+	−	−	+	−	−	−	+	+	+	+	−	−	−
D-Glucose	v!	v!	+	v!	+	v!	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	v	+	+	+	+	−	v	+	+	v	v	−	+	v	−	+	+	+	(+)	(+)	+
L-Fucose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
D-Lactose	+	+	+	+	+	+	+	+	v!	(+)	+	−	+	−	+	+	+	+	+	−	−
D-Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−
L-Rhamnose	+	v	−	−	v	−	−	+	+	−	+	−	+	+	+	v	+	+	+	+	+
D-Ribose	−	−	−	+	−	−	+	+	−	+	v	+	+	+	−	+	v	+	−	+	+
D-Saccharose (Sucrose)	+	+	+	+	+	−	−	−	−	−	−	−	v	−	−	−	−	+	−	−	−
Methyl α-D-glucoside	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+	−
Methyl α-D-mannose	−	−	nd	−	nd	−	+	−	−	−	−	−	v	−	−	−	−	+	(+)	−	−
Potassium 5-ketogluconate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
D-Xylose	−	−	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	−	−	−	−	−	−	−	−	−	v	+	−	−	+	+	+	+	−	−	−	+
Glucose 1-phosphate	−	−	−	v	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−	−	v	−	v	v	−	−	−	−	−	+	+

Inulin	v!	v!	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-Lyxose	v	v	–	–	v	–	v	–	–	–	–	–	–	v	+	–	–	–	–	–	+
D-Mannitol	–	–	–	–	–	–	+	+	+	–	v	–	v	–	–	+	+	–	–	–	–
D-Melezitose	v	v	v	v	v	–	–	–	–	–	–	–	v	–	–	–	–	–	–	–	–
D-Melibiose	v!	v	–	–	–	v	–	+	–	–	v	–	v	–	–	–	+	–	–	–	–
L-Sorbose	v!	v!	–	v!	–	v!	v!	–	–	–	–	–	v	–	–	–	–	–	–	–	–
D-Tagatose	–	–	–	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	v
D-Turanose	–	v	–	–	–	+	–	–	–	–	–	–	v	–	–	–	–	–	–	–	–

Notation: +, positive; (+), weakly positive; −, negative; v, variable (between replicates and/or between strains); v!, variable between studies (possibly due to differences in incubation times and temperatures between studies); *, all *L. valentina* strains, based on API 50 CH, produce acid from gentiobiose and D-trehalose but not produce acid from dulcitol, L-arabitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, potassium gluconate, xylitol, starch, erythritol, D-sorbitol, L-xylose; **, based on API *Listeria* (negative based on API 50 CH); nd , not determined or not recorded.

Strains: *Lmo*, *L. monocytogenes* strain 10403S (data from [2] and [12]); *Lin, L. innocua* strain FSL S4-378 (data from [2] and [12]); *Lse, L. seeligeri* (data from [2] and [12]); *Liv, L. ivanovii* strain ATCC BAA-678 (data from [2] and [12]); *Lws, L. welshimeri* (data from [2] and [12]); *Lma, L. marthii* strain FSL S4-120 T (data from [15]); *Lgy, L. grayi* strains ATCC 19120 T, ATCC 25401 T (data from [15]); *Lro, L. rocourtiae* strain CIP 109804 T (data from [15]); Lwp, L. weihenstephanensis strain DSM 24698 T (data from [15]); *Lcn, L. cornellensis* strains TTU A1-0210 T, FSL F6-0970 (data from [15]); *Lri, L. riparia* strains FSL S10-1204 T, FSL S10-1219 (data from [15]); *Lgd, L. grandensis* strain TTU A1-0212 T (data from [15]); *Lfc, L. fleischmannii* strains DSM 24998T, ATCC BAA-2414 T, FSL F6-1019, FSL S10-1186, FSL S10-1203 and FSL S10-1220 (data from [15]); *Laq, L. aquatica* strains FSL S10-1188 T and FSL S10-1181 (data from [15]); *Lfo, L. floridensis* strain FSL S10-1187 T (data from [15]); *Lny, L. newyorkensis* strains FSL M6-0635 T and A5-0209 (data from [16]); *Lbo, L. booriae* strains FSL A5-0279 T and FSL A5-0281(data from [16]); *Lco, L. costaricensis* strain CIP 111400T (data from [17]); *Lgo, L. goaensis* strain DSM 29886 (data from [18]); *Lth, L. thailandensis* strain CLIP 2015/00305T (data from [19]); *Lva, L. valentina* sp. nov. strains CLIP 2019/00642^{T} , CLIP 2019/00569, CLIP 2019/00570, CLIP 2019/00571, CLIP 2019/00572 and CLIP 2019/00574 (this study).

All species/strains are positive for aesculin and acid production from N-acetylglucosamine, amygdalin, arbutin, D-cellobiose, D-fructose, D-mannose and salicin.

All species/strains are negative for nitrite reduction and acid production from D-adonitol, D-arabinose, glycogen, methyl β-D-xylopyranoside, potassium 2-ketogluconate, and D-raffinose.

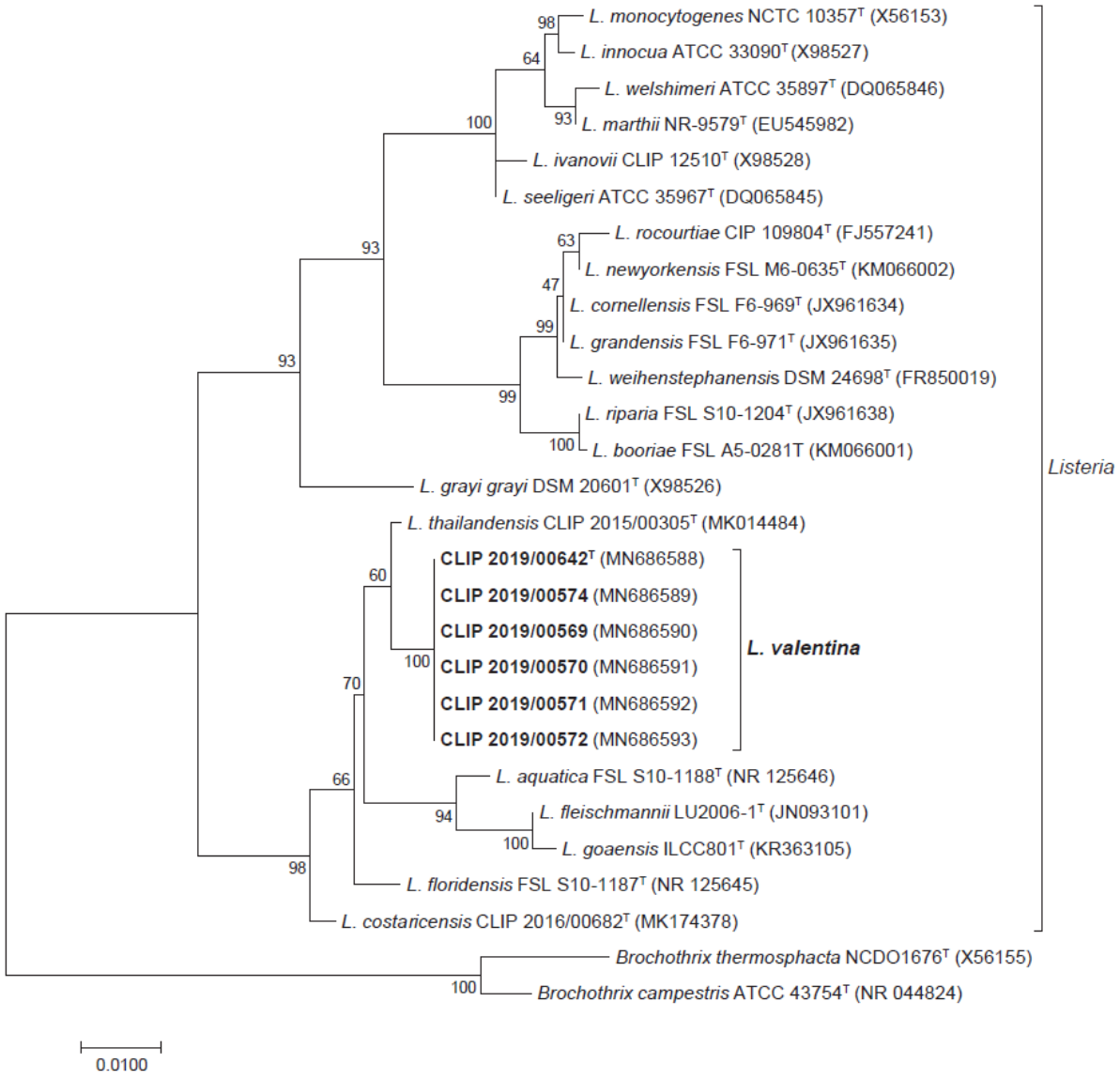


FIGURE 1. Phylogenetic analysis of the 16S rRNA gene based on the maximum likelihood method. Distance estimation was obtained by the model of Kimura 2-parameter [37]. Selected members of *Brochothrix* genus were used as outgroup. Positions containing gaps and missing data were eliminated, resulting in a total of 1072 positions. Branch lengths represent the number of nucleotide substitutions per site and bootstrap percentages of 1,000 replicates are shown. GenBank accession numbers are provided in brackets. The newly *Listeria valentina* isolates is highlighted in bold**.**

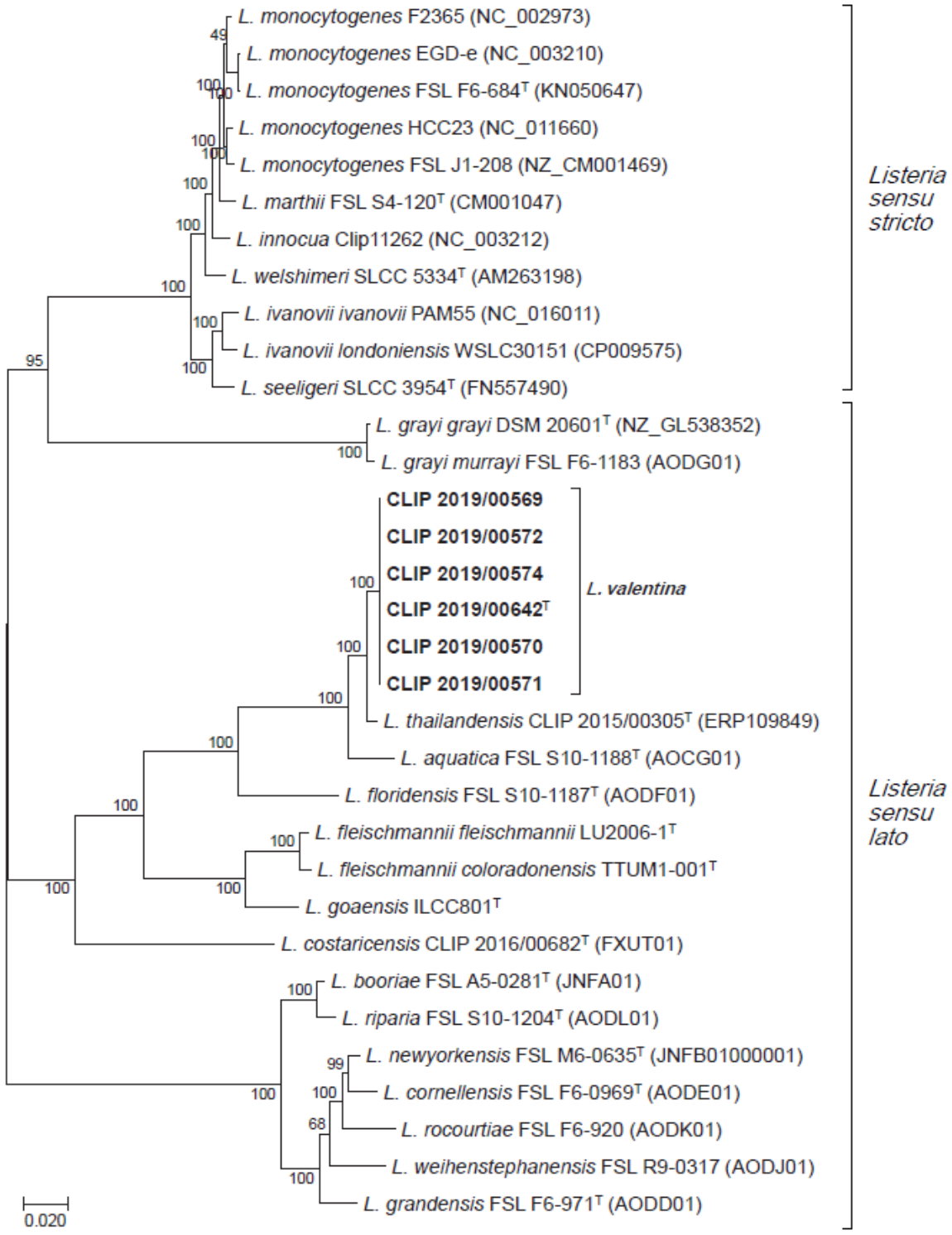


FIGURE 2. Maximum likelihood phylogenetic analysis based on the concatenated amino acid sequences of 231 core genes present in all *Listeria* species. Distance estimation was obtained by the Whelan and Goldman (WAG) model [38]. Branch lengths represent the number of amino acid substitutions per site and bootstrap percentages of 1,000 replicates are shown. GenBank accession numbers are provided in brackets. *Listeria valentina* isolates are highlighted in bold**.**

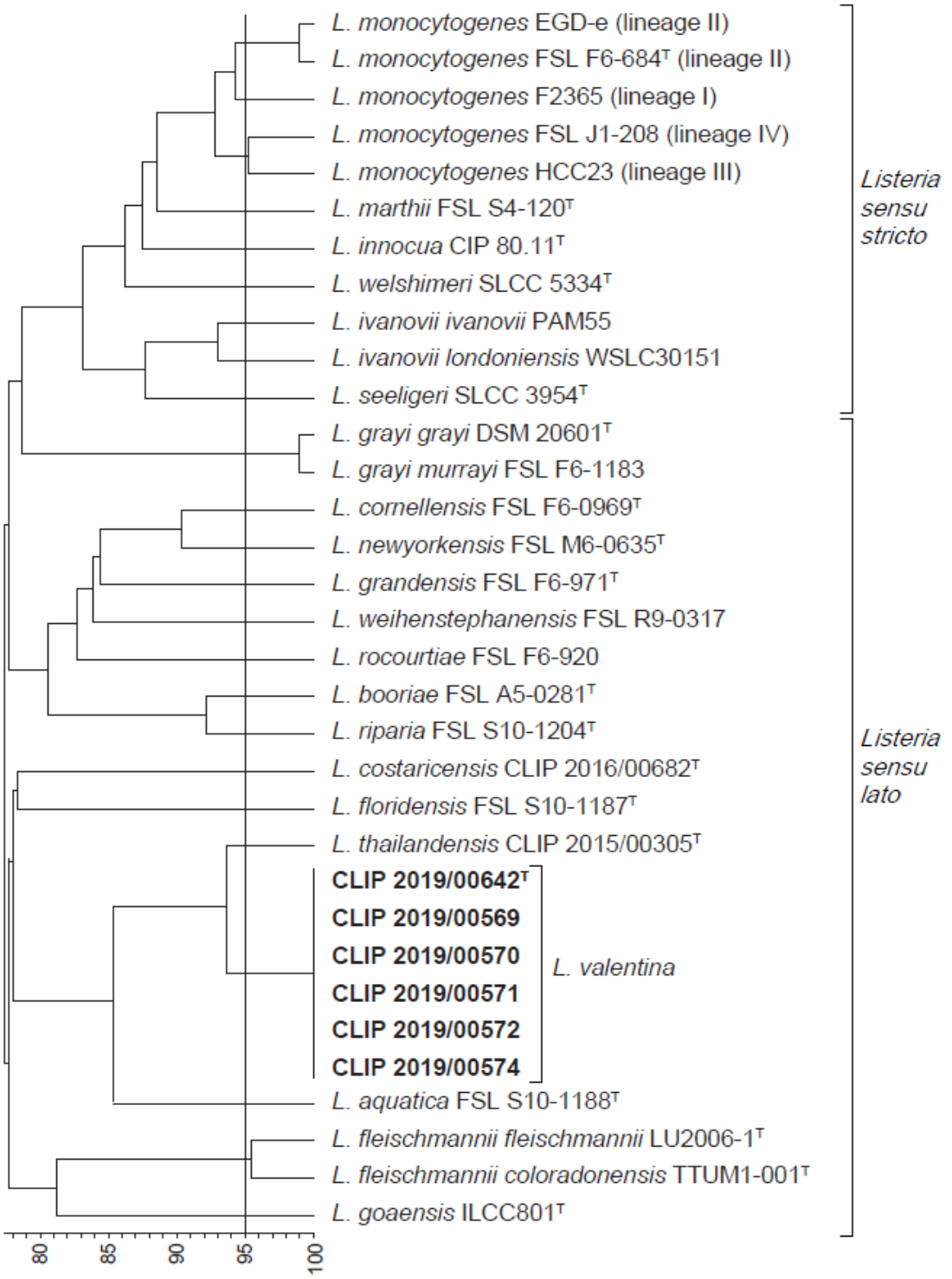
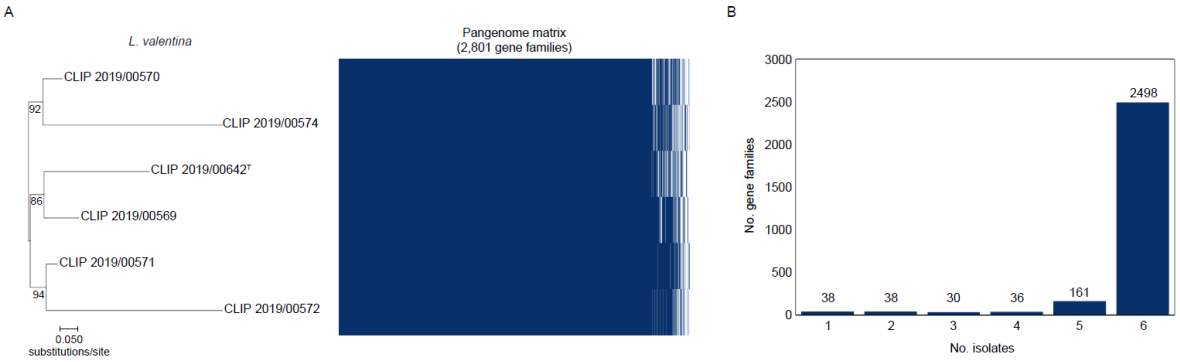


FIGURE 3. UPGMA clustering based on the genomic average nucleotide difference (ANI). The vertical dashed bar represents the proposed 95% ANIb species cut-off that correlates with the 70% DNA-DNA hybridization threshold [43]. Scale bar represents the percentage of similarity. *Listeria valentina* type strain is highlighted in bold**.**



Supplementary Figure 1. Pangenome analysis of *L. valentina* sp. nov. isolates. A) Presence (blue) and absence (white) patterns of the 2,801 gene families in each isolate, as determined by Roary (Page *et al.*, 2015), using a 95% BLASTP identity cut-off. A midpoint rooted recombination-free phylogeny built from the *L. valentina* genome alignment (2,536,866 nucleotide positions) using Parsnp (Treangen *et al.*, 2014) is shown on the left. Numbers at the nodes indicate the bootstrap values based on 1,000 replicates. B) Frequency plot of the gene families *versus* the number of isolates.

Supplementary Figure 2. UPGMA clustering using of the 33 biochemical characteristics of *Listeria* species (shown in Table 1), using the Dice similarity coefficient. Black and white boxes represent the presence and absence of phenotypes, respectively. Unknown data (marked as ”ND”) and traits with variations between different isolates and/or studies (gray boxes) were ignored in the analysis. Scale bar represents the percentage of similarity between phenotypical profiles.